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Oxidative burst inhibitory and cytotoxic amides and lignans from the stem bark of *Fagara heitzii* (Rutaceae)

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ABSTRACT

Two amides, heitziamide A and heitziamide B and two phenylethanoids, heitziethanoid A and heitziethanoid B together with thirteen known compounds were isolated from *F. heitzii* (Letouzey). The structures of all compounds were established by spectroscopic analysis.

Nine compounds were evaluated for oxidative burst inhibitory activity in a chemoluminescence assay and for cytotoxicity against PC-3 prostate cancer cells.

All compounds exhibited a clear suppressive effect on phagocytosis response upon activation with serum opsonized zymosan at the range of IC_{50} = 2.0–6.5 μM, but no cytotoxic effect was observed (IC_{50} > 100 μM).

1. Introduction

Immune suppression and cytotoxic activity affecting the function of the immune system have been reported for many synthetic and natural therapeutic agents (Asuman and Barlas, 1984; Sell, 1987; Siddiqui et al., 2001). Various conditions such as infections, organ transplantation, cancer, rheumatoid arthritis, and systemic lupus erythromatosus are currently treated with novel immunomodulating agents (Bartlett et al., 1991).

As part of a program to discover potential antitumoral, anti-inflammatory, antioxidant, and antimalarial agents from Cameroonian rainforest medicinal plants, we investigated *Fagara heitzii* (syn. *Zanthoxylum heitzii*) (Rutaceae). This species is a shrub of the humid rain forests of Cameroon, where it is traditionally used as medicinal plant against cancer, syphilis, malaria, cardiac palpitations, urogenital affections as well as to prepare poisonous arrows (Sandberg et al., 2005; Adjanohoon et al., 1988; Zirihi et al., 2005). Previous phytochemical investigations of *F. heitzii* have yielded methylnitidine chloride, nitidine, fagaramide, flindersine, lignans, terpenes and steroids (Bongui et al., 2005; Ngouela et al., 1994; Saboor, 1984). In this paper, we report the isolation, the structure elucidation of four new compounds (**1**–**4**), and their biological activities.

2. Results and discussion

The stem bark of *F. heitzii* was extracted with MeOH. The extract was separated using repeated column chromatography and preparative TLC (PTLC) to afford four new compounds (**1**–**4**) and thirteen known compounds (Fig. 1). By comparison with the reported data (Mbaze et al., 2007; Chaaib et al., 2003; Ayafor et al., 1984), the known compounds were identified as *trans*-fagaramide (**5**), arnottianamide (**6**), iso-γ-fagarine (**7**), iso-skimmianine (**8**), arctigenin methyl ether (**9**), savinin (**10**), (+)-eudesmin (**11**), (+)-sesamin (**12**), lupeol, lupeone, β-sitosterol, stigmasterol and stigmasterol-3-*O*-β-*D*-glucopyranoside.

Heitziamide A (**1**) was obtained as a white amorphous powder. Its molecular formula $C_{24}H_{33}NO_3$ was deduced from EI-MS and HR-EI-MS ($[M]^+$, *m*/*z* found 383.2452, calc. 383.2460). The UV spectrum showed two maxima at 260 and 302 nm, suggesting the presence of an amide group. The presence of an amide function was

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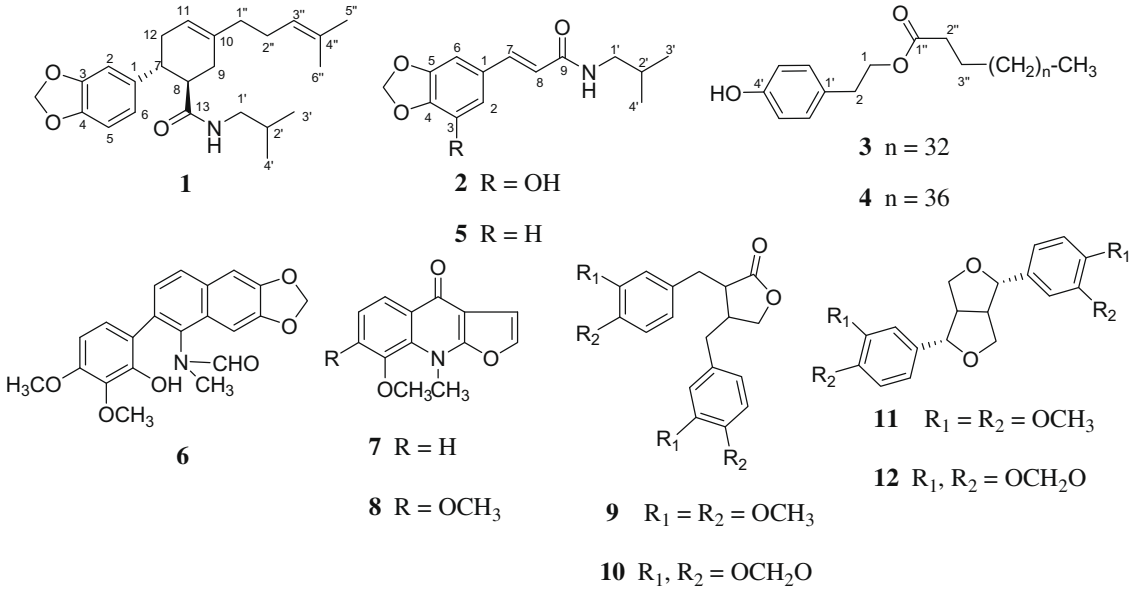

Fig. 1. Structures of some isolated compounds.

Table 1
^{1}H (500 MHz) and ^{13}C (125 MHz) NMR assignments for **1** and **2** in $CDCl_3$.

Attribution	**1**		**2**	
	^{13}C	^{1}H	^{13}C	^{1}H
1	138.3	–	129.3	–
2	108.3	6.74 (*d*, *J* = 1.3)	106.3	7.12 (*s*)
3	147.6	–	140.6	–
4	146.0	–	135.9	–
5	107.6	6.72 (*d*, *J* = 8.2)	148.2	–
6	120.8	6.70 (*dd*, *J* = 8.2; 1.3)	101.4	6.80 (*s*)
7	42.8	2.92 (*ddd*, *J* = 13.2; 11.3; 5.6)	141.2	7.55 (*d*, *J* = 15.4)
8	49.4	2.43 (*td*, *J* = 11.3; 5.0)	118.8	6.23 (*d*, *J* = 15.4)
9	33.6	2.16 (*m*)	167.1	
10	136.3	–		
11	119.7	5.49 (*brs*)		
12	32.7	2.25 (*m*)		
13	174.4	–		
1′	46.6	2.96 (*m*) 2.69 (*dd*, *J* = (6.5; 5.0)	46.9	3.24 (*t*, *J* = 6.3)
2′	28.3	1.43 (*m*)	28.7	1.87 (*m*)
3'	19.8	0.68 (*d*, *J* = 6.3)	20.1	0.96 (*d*, *J* = 6.6)
4′	19.8	0.66 (*d*, *J* = 6.3)	20.1	0.96 (*d*, *J* = 6.6)
1″	26.4	2.23 (*m*)		
2″	37.4	2.04 (*m*)		
3″	124.1	5.13 (*t*, *J* = 6.9)		
4″	131.7	–		
5″	17.7	1.63 (*s*)		
6″	25.7	1.71 (*s*)		
–OCH_2O–	100.8	5.90 (*d*, *J* = 1.3) 5.91 (*d*, *J* = 1.3)		6.01 (*brs*)
NH		5.21 (*t*, *J* = 5.0)		5.61 (*brs*)
OH				9.80 (*brs*)

Assignments were based on HMQC, HMBC and NOESY experiments.

further indicated by two IR bands at 3289 and 1629 cm^{-1}. The ^{1}H NMR spectrum (Table 1) of compound **1** exhibited signals for methylenedioxy protons at δ 5.90 (*d*, *J* = 1.3 Hz) and 5.91 (*d*, *J* = 1.3 Hz), and an ABX pattern of three aromatic protons at δ 6.70 (*dd*, *J* = 8.2; 1.3 Hz, 1H, H-6); 6.72 (*d*, *J* = 8.2 Hz, 1H, H-5) and 6.74 (*d*, *J* = 1.3 Hz, 1H, H-2). These findings are in agreement with the presence of a methylenedioxyphenyl group in compound **1**. This assumption was supported by the ^{13}C NMR (Table 1), DEPT and EI-MS spectra which show a characteristic signal of methylenedioxy at δ 100.8, six aromatic carbons at δ 107.6, 108.3, 120.8, 138.3, 146.0 and 147.6. Furthermore, the ^{1}H NMR spectrum showed signals characteristic of the cyclohexene ring at δ 2.16–2.24 (*m*, 4 H, H-9 and H-12); 2.43 (*td*, *J* = 11.3, 5.0 Hz, 1H, H-8); 2.92 (*ddd*, *J* = 13.2, 11.3, 5.6 Hz, 1H, H-7) and 5.49 (*brs*, 1H, H-11) (Joshi et al., 1975). The cyclohexene ring was confirmed by the ^{1}H–^{1}H COSY spectrum, in which several cross-peaks were observed; correlation between proton H-11 (δ 5.49) and the methylene H-12 (δ 2.16–2.25), between H-12 and H-7 (δ 2.92), and finally between H-7 and H-8 (δ 2.43), which in turn was also correlated to the methylene H-9 (δ 2.16-2.25). The ^{1}H NMR spectrum also exhibited signals of an isohexenyl group at δ 1.63 (*s*, 3H, H-5″); 1.71 (*s*, 3H, H-6″); 2.04 (*m*, 2H, H-2″); 2.23 (*m*, 2H, H-1″) and 5.13 (*t*, *J* = 6.9 Hz 1H, H-3″), and of an isobutylamide at δ 0.66 (*d*, *J* = 6.3 Hz, 3H, H-4′); 0.68 (*d*, *J* = 6.3 Hz, 3H, H-3′); 1.43 (*m*, 1H, H-2′); 2.69 (*dd*, *J* = 6.5, 5.0 Hz, 1H, H-1′a); 2.96 (*m*, 1H, H-1′b) and 5.21 (*t*, *J* = 5.0 Hz, NH) (Portet et al., 2007). The presence of isohexenyl and isobutylamide moieties was further confirmed by the ^{13}C NMR, DEPT and HMQC spectra, which showed two methyls, three methylenes, two methines, one amide (δ 174.4), and one quaternary carbon (δ 131.7).

To establish the connections between the above-described structural units, an HMBC analysis was done (Fig. 2). In the HMBC spectrum, correlations for the proton H-7 (δ 2.92) to carbons C-1 (δ 138.3), C-6 (δ 120.8), C-2 (δ 108.3), C-13 (δ 174.4), C-11 (δ 119.7), and C-9 (δ 33.6) and for the proton H-8 (δ 2.43) to carbons C-1(δ 138.3), C-13 (δ 174.4), C-10 (δ 136.3), and C-12 (δ 32.7) established the attachment of the methylenedioxyphenyl, the isohexenyl and the isobutylamide moieties to the cyclohexene ring at C-7, C-10, and C-8, respectively. The relative configurations of the two stereogenic centers of **1** were elucidated on the basis of the ^{1}H–^{1}H coupling constants and NOESY correlations as shown in Fig. 3. The coupling constant of H-7/H-8 (*J* = 11.3 Hz) and the lack of the NOESY correlation between the two protons indicated the anti relationship of H-7/H-8 and their axial orientations (Wei et al., 2005). Furthermore, since the optical rotation was zero and the circular dichroism (CD) spectrum exhibited no Cotton effect; compound **1** was concluded to be a racemate. From the above spectroscopic studies, therefore 1 is (±) 6-(3,4-methylenedioxyphenyl)-*N*-isobutyl-3-(isohexenyl)cyclohex-3-enecarboxamide named as heitziamide A.

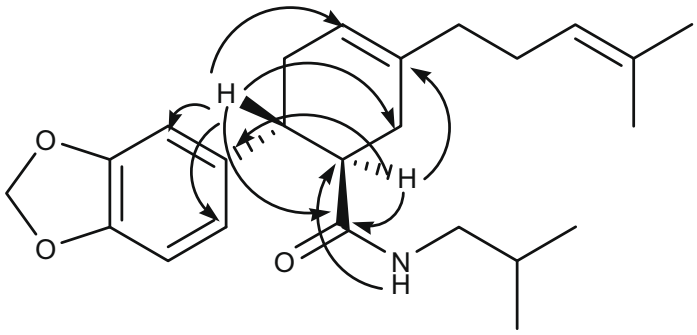

Fig. 2. Important HMBC correlations for **1**.

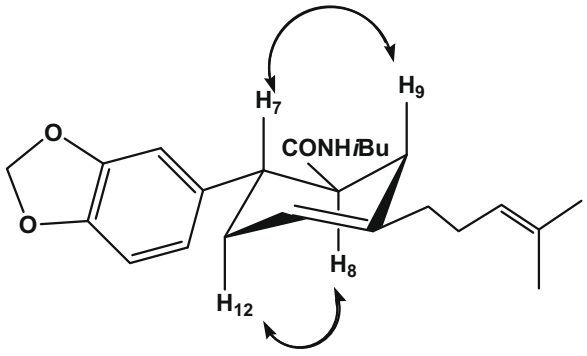

Fig. 3. Selected NOESY correlations for **1**.

This compound is a novel cyclohexene-type amide. We propose a possible biosynthesis for its formation based on closer examination of the structure. This compound could result from the intermolecular [4 + 2] cycloaddition reaction (Diels–Alder reaction) between the monoterpene 6-methyl-2-vinylheptane-1,5-diene (**13**) (which results from two isoprenyl units linked by head/tail) and the *trans*-fagaramide (**5**) (Fig. 4). The *trans*-stereochemistry of the product postulated in the biosynthetic hypothesis was identical to that of the natural compound **1**.

Heitziamide B (**2**) was obtained as a yellow amorphous powder. Its molecular composition was $C_{14}H_{17}NO_4$ as shown by EI-MS and HR-EI-MS ([M] $^+$, *m*/*z* 263.11410, calc. 263.11581). The UV spectrum exhibited several absorption maxima at 325, 290, 235, and at 215 nm. The presence of hydroxyl and amide functions was indicated by two IR bands at 3350 and 1680 cm^{-1}, respectively. The ^1H NMR spectrum (Table 1) of compound **2**, exhibited signals for methylenedioxy protons at δ 6.01 (*brs*, 2H), two aromatics protons appeared as singlet at δ 7.12 (*brs*) and 6.80 (*brs*), and one hydroxyl group at δ 9.80 (*brs*) exchangeable with D_2O. These findings are in agreement with the presence of a hydroxylmethylenedioxyphenyl group in compound **2**. This assumption was further supported by the ^{13}C NMR (Table 1) and DEPT spectra, which show the characteristic signal of the methylenedioxy group at δ 101.3, together with six aromatic carbons at δ 101.4, 106.3, 129.3, 135.9, 140.6, and 148.2.

Furthermore, the ^1H NMR spectrum showed signals characteristic for the isobutylamide at δ 0.96 (*d*, *J* = 6.6 Hz, 6H, H-3′and H-4′); 1.87 (*m*, 1H, H-2′); 3.24 (*t*, *J* = 6.3 Hz, 2H, H-1′) and 5.61 (*brs*, NH) (Kubo et al., 1984), and an AB system of two olefinic protons at δ 6.23 (*d*, *J* = 15.4 Hz, 1H, H-8) and 7.55 (*d*, *J* = 15.4 Hz, 1H, H-7). The large coupling constant (*J* = 15.4 Hz) between H-7 and H-8 indicated a *trans* configuration at the double bond. All these above-described structural units were confirmed by the ^{13}C NMR, DEPT, COSY, HMQC, and HMBC spectra, which showed characteristic signals of *trans*-fagaramide (**5**) (Okorie, 1976; Kubo et al., 1984). The absence of an ABX system of three aromatic protons in compound **2**, suggested that one of the aromatic carbons was hydroxylated. This assumption was further supported by EIMS (*m*/*z* 263), which was 16 mass units higher than that of *trans*-fagaramide (**5**) (*m*/*z* 247) confirming the presence of additional oxygen in compound **2**. The location of the hydroxyl group at C-3 was based on the correlations for H-7 (δ 7.55) to C-2 (δ 106.3), C-6 (δ 101.4) and C-9 (δ 167.1) as observed in HMBC.

From the above spectroscopic studies, the structure of compound **2** was assigned as *trans*-3-hydroxyfagaramide named heitziamide B.

Heitziethanoid A (**3**) and B (**4**) were obtained as white amorphous powders, showing positive reactions with $FeCl_3$ indicating their phenolic nature. Their UV spectrum exhibited two absorption maxima at 275 and 240 nm characteristic of phenylethanoids (Chaaib et al., 2003; Chihiro et al., 1988). The presence of hydroxyl and ester functions was indicated by two IR bands at 2935 and 1750 cm^{-1}, respectively. From the HR-ESI-MS, the pseudomolecular ion was found to be $C_{44}H_{80}O_3Na$ by ([M+Na]$^+$ at *m*/*z* = 679.6010, calc. 679.6005) and $C_{48}H_{88}O_3Na$ by ([M+Na]$^+$ at *m*/*z* = 735.6649, calc. 735.6631), respectively.

The ^1H NMR spectra of **3** and **4** showed the typical AA'XX' system of a *p*-disubstituted benzene ring at δ 7.06 (*d*, *J* = 8.3 Hz, H-2′, H-6′) and 6.80 (*d*, *J* = 8.3 Hz, H-3′, H-5′), the presence of an ethyl alcohol unit at δ 4.23 (*t*, *J* = 6.9 Hz, H-1), and 2.84 (*t*, *J* = 6.9 Hz, H-

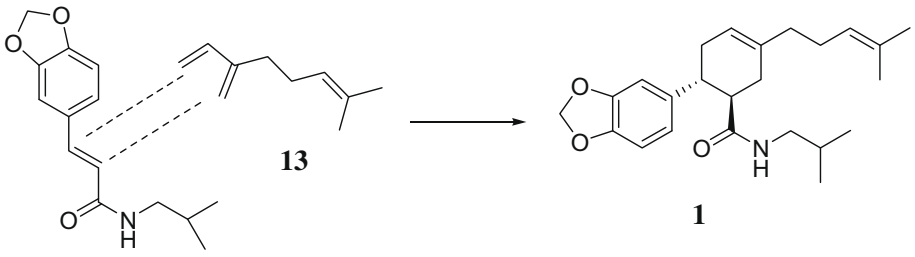

Fig. 4. Biosynthesis hypothesis of compound **1**.

2), and a free hydroxyl group at δ 9.58 (*brs*, OH-4′) exchangeable with D_2O. This inference was supported by the ^{13}C NMR and DEPT data, which showed characteristic signals of a *p*-disubstituted benzene ring at δ 114.4 (C-3′, C-5′), 129.1 (C-1′), 130.2 (C-2′, C-6′) and 156.7 (C-4′), and ethyl alcohol unit at δ 65.2 (C-1) and 34.3 (C-2) (Acevedo et al., 2000). Furthermore, in the ^1H NMR spectrum, a terminal methyl at δ 0.90 (*t*, *J* = 6.8 Hz) and methylenes at δ 2.29 (*t*, *J* = 7.5 Hz, H-2″), 1.58 (*m*, H-3″) 1.25 (*brs*, nH) were also observed. These data suggested the presence of a long alkyl chains linked to the 4-hydroxyphenylethanol moiety. The presence of a long alkyl chains was further confirmed by ^{13}C NMR spectrum, which showed characteristic signals at δ 174.0 (C-1″), 34.1 (C-2″), 31.8-29.3 $(CH_2)_n$ 24.8 (C-3″) and 14.0 (CH_3) (Wandji et al., 2002).

To determine the linkage between the long chain and the 4-hydroxyphenylethanol moiety, HMBC experiment was performed. In the HMBC spectrum, correlation of H-1 (δ 4.23) with C-1″ (δ 174.0), C-1′ (δ 129.1) and C-2 (δ 34.1), suggested that the long chain is linked to the 4-hydroxyphenylethanol moiety by an ester function.

The methanolysis of **3** and **4** yielded methyl hexatriacontanoate (identified by the ESIMS indicating a pseudomolecular ion at *m/z* 573 $[M+Na]^+$, corresponding to a molecular formula $C_{37}H_{74}O_2$), methyl tetracontanoate (identified by the ESIMS indicated a pseudomolecular ion at *m/z* 629 $[M+Na]^+$, corresponding to a molecular formula $C_{41}H_{82}O_2$) and 4-hydroxyphenylethanol. 4-hydroxyphenylethanol was identified by the ^1H NMR and EI-MS. From the above spectroscopic studies, heitziethanoid A (**3**) and B (**4**) were characterized as 2-(4-hydroxyphenethyl)hexatriacontanoate and 2-(4-hydroxyphenethyl)tetracontanoate, respectively. Phenylethanoids have previously been described in the genus *Citrus* and *Fagara* (Rutaceae) (Chaaib et al., 2003; Chihiro et al., 1988).

Compounds **1**, **2**, **5**, and **7**–**12** were screened over a wide range of concentrations (3.1–50 μg ml^{-1}) for their immunomodulatory potential. These compounds were shown to possess inhibitory activity upon activation with serum opsonized zymosan, which was tested *in vitro* for oxidative burst studies of whole blood. All these compounds showed significant effects on the oxidative burst of the whole blood (IC_{50} range 2.0–6.5 μM) compared to the control (Ibuprofen IC_{50} = 12.1 μM) (Table 3). The tested compounds exhibited a clear suppressive effect on phagocyte oxidative burst response upon activation with serum opsonized zymosan in a dose-dependent manner.

In addition, all these compounds were evaluated for their cytotoxicity against prostate cancer cell (PC-3) cell line using MTT method and doxorubicin as positive control. However, no cytotoxic effect was observed on prostate cancer cell (IC_{50} > 100 μM).

Table 2
^1H (500 MHz) and ^{13}C (125 MHz) NMR assignments for **3** and **4** in $CDCl_3$ + MeOD.

Attribution	**3**–**4**	
	^{13}C	^1H
1	65.2	4.23 (*t*, *J* = 6.7)
2	34.3	2.84 (*t*, *J* = 6.7)
1′	129.6	–
2′/6′	130.2	7.06 (*d*, *J* = 8.4)
3′/5′	114.4	6.80 (*d*, *J* = 8.4)
4′	156.7	–
1″	174.0	–
2″	34.1	2.29 (*m*)
3″	24.8	1.58 (*m*)
$-(CH_2)_n-$	29.4	1.25 (*brs*)
CH_3-	14.0	0.90 (*t*, *J* = 6.8)
[a]OH		9.58 (*brs*)

[a] Observed in DMSO.

Table 3
Effect of compounds **1**, **2**, **5**, and **7**–**10** on oxidative burst of whole blood.

Compounds	IC_{50} (μM)
1	2.6 ± 0.4
2	2.0 ± 0.1
5	3.4 ± 0.4
7	3.1 ± 0.3
8	2.3 ± 0.2
9	6.5 ± 0.5
10	5.6 ± 0.8
11	6.0 ± 0.5
12	2.2 ± 0.3
Ibuprofen[a]	12.1 ± 3.0

[a] Standard used in the assay.

Since these compounds are weakly toxic and exhibited a clear suppressive effect on phagocyte oxidative burst, they can be therefore considered as potential antioxidants drugs.

3. Experimental section

3.1. General

Optical rotations $[\alpha]_D$ (MeOH, *c* in g ml^{-1}) were determined by using a JASCO digital polarimeter (model DIP-3600). Infrared spectra were recorded on a JASCO FT/IR-410 spectrophotometer. UV spectra were determined on a Spectronic Unicam spectrophotometer. HR-ESI-MS were recorded on a APEX III (Bruker Daltonik) 7 Tesla (ESI FT-ICR-MS). EI-MS spectra were recorded on a Finnigan MAT 95 spectrometer (70 eV) with perfluorokerosene as reference substance for HR-EI-MS. The ^1H and ^{13}C NMR spectra were recorded at 500 MHz and 125 MHz, respectively, on Bruker AMX 500 NMR spectrometers. Methyl, methylene and methine carbons were distinguished by DEPT experiments. Homonuclear ^1H connectivities were determined by using COSY experiments. One bond ^1H–^{13}C connectivities were determined with HMQC gradient pulse factor selection. Two- and three- bond ^1H–^{13}C connectivities were determined in HMBC experiments. Chemical shifts are reported in δ (ppm) using TMS as internal standard and coupling constants (*J*) were measured in Hz. Column chromatography was carried out on silica gel (70–230 mesh, Merck) and flash silica gel (230–400 mesh, Merck). TLC was performed on Merck precoated silica gel 60 (F_{254}, Merck), and spots were visualized by using ceric sulfate spray reagent. All reagents used were of analytical grades.

3.2. Plant material

The stem bark of *F. heitzii* Letouzey was collected at Mbalmayo locality, Centre, Cameroon in June 2006 and identified by Mr. Nana Victor of National Herbarium, Yaounde, Cameroon, where the voucher specimen (Ref. No. 1482/SRFK) has been deposited.

4. Extraction and isolation

The powdered stem bark of *F. heitzii* (4.5 kg) was extracted with MeOH at room temperature during 72 h. After removing the solvents by evaporation under reduced pressure, the obtained crude extract (110.0 g) was chromatographed over silica gel 60 (70–230; 230–400 mesh), using hexane, ethyl acetate, and MeOH in increasing polarity order. A total of 162 sub-fractions (ca. 250 ml each) were collected and combined on the basis of TLC analysis leading to five main fractions A, B, C, D, and E.

Fraction A (28.0 g, combined from sub-fractions 1–33) was chromatographed on silica gel with a hexane–ethyl acetate gradi-

ent to yield lupeol (30.6 mg), β-sitosterol (19.6 mg) and stigmasterol (16.1 mg).

Fraction B (32.0 g, combined from sub-fractions 34–98) was chromatographed on silica gel with a hexane–ethyl acetate gradient. A total of 35 fractions (ca. 75 ml each) was collected and combined on the basis of TLC. Fractions (7–35) were further chromatographed on silica gel with a mixture hexane–ethyl acetate (3:1) to yield lupeone (17.0 mg), savinin (**10**) (71.3 mg), (+)-eudesmin (**11**) (11.0 mg), (+)-sesamin (**12**) (19.6 mg) and arctigenin methyl ether (**9**) (8.0 mg).

Fraction C (130.0 g, combined from sub-fractions 99–113) was chromatographed on silica gel with a hexane–ethyl acetate gradient. A total of 32 fractions (ca. 100 ml each) was collected and combined on the basis of TLC. Fractions (6–32) were further chromatographed on silica gel with a mixture of hexane–ethyl acetate (2:1) to yield arnottianamide (**6**) (16.6 mg), *trans*-fagaramide (**5**) (94.4 mg), iso-γ-fagarine (**7**) (10.3 mg) and iso-γ-skimmianine (**8**) (9.1 mg).

Fraction D (21.8 g, combined from sub-fractions 114–140) was chromatographed on silica gel with a hexane – ethyl acetate gradient. A total of 41 fractions (ca. 100 ml each) was collected and combined on the basis of TLC. Fractions 3–41 were further chromatographed on silica gel to yield heitziamine A (**1**) (12.2 mg), heitziamine B (**2**) (9.0 mg), heitziethanoid A (**3**) (11.9 mg) and heitziethanoid B (**4**) (18.3 mg).

Fraction E (11.0 g, combined from sub-fractions 141–162) was chromatographed on silica gel and eluted with a mixture of ethyl acetate–MeOH of increasing polarity. A total of 33 fractions (ca. 100 ml each) was collected and combined on the basis of TLC. Fractions 1–13 were further chromatographed on silica gel with a mixture to yield stigmasterol-3-*O*-β-*D*-glucopyranoside (24.6 mg).

4.1. Heitziamide A (1)

Amorphous powder; $[\alpha]_D^{25} = 0$ (c = 0.010 in $CHCl_3$); UV λ_{max}^{MeOH} (log ε) nm: 302 (4.82), 260 (3.86); IR (KBr) ν_{max}: 3310, 3289, 2920, 1660, 1629, 1585, 1540, 920 cm^{-1}; ^1H and ^{13}C NMR see Table 1; HR-EI-MS m/z 383.24520 (calc. for $C_{24}H_{33}NO_3$, 383.24336); EI-MS m/z (rel. int.): 383 (100) $[M]^+$, 314 (31), 283 (22), 247 (33), 191 (5), 176 (5), 148 (42), 136 (12), 135 (75), 69 (54).

4.2. Heitziamide B (2)

Yellow amorphous powder; UV λ_{max}^{MeOH} (log ε) nm: 325 (3.41), 290 (3.04), 235 (2.46), 215 (2.25); IR (KBr) ν_{max}: 3350, 3178, 2920, 1680, 1620, 1581, 925 cm^{-1}; ^1H and ^{13}C NMR see Table 1; HR-EI-MS m/z 263.11410 (calc. for $C_{14}H_{17}NO_3$, 263.11581); EI-MS m/z (rel. int.): 263 (12) $[M]^+$, 247 (25), 190 (90), 175 (18), 163 (20), 151 (28), 145 (55), 117 (45).

4.3. Heitziethanoid A (3)

White amorphous powder; UV λ_{max}^{MeOH} (log) nm: 275 (3.8), 240 (3.7); IR ($CHCl_3$) ν_{max}: 2935, 2860, 1750, 1616, 1390 cm^{-1}; ^1H and ^{13}C NMR see Table 2; HR-ESI-MS m/z 656.6010 (calc. for $C_{44}H_{80}O_3Na$, 679.6005); ESI–MS m/z = 679 $[M + Na]^+$.

4.3.1. Methyl hexatriacontanoate

Colourless oil; ESI-MS m/z = 573 $[M+Na]^+$; ^1H NMR (500 MHz, $CDCl_3$): δ 0.73 (*t*, *J* = 7.6 Hz, 3H, $-CH_3$), 1.42–1.10 (*brs*, $-(CH_2)_n-$), 2.08 (*t*, *J* = 7.9 Hz, 2H, H-2), 3.80 (*s*, 3H, $-OCH_3$).

4.4. Heitziethanoid B (4)

White amorphous powder; UV λ_{max}^{MeOH} (log ε) nm: 277 (3.8), 240 (3.6); IR ($CHCl_3$) ν_{max}: 2935, 2850, 1750, 1618, 1387 cm^{-1}; ^1H and ^{13}C NMR see Table 2; HR-ESI-MS m/z 735.6649 (calc. for $C_{48}H_{88}O_3Na$, 735.6631); ESI–MS m/z = 735 $[M+Na]^+$.

4.4.1. Methyl tetracontanoate

Colourless oil; ESI–MS m/z = 629 $[M+Na]^+$; ^1H NMR (500 MHz, $CDCl_3$): δ 0.74 (*t*, *J* = 7.5 Hz, 3H, $-CH_3$), 1.42–1.12 (*brs*, $-(CH_2)_n-$), 2.10 (*t*, *J* = 8.0 Hz, 2H, H-2), 3.82 (*s*, 3H, $-OCH_3$).

5. Chemical derivatives

Methanolysis of compounds (**3**–**4**): Compounds **3**–**4** (5.0 mg each) were added to a mixture of HCl (3.5 ml, 1 N) and dry MeOH (6.0 mg), and refluxed for 16 h with magnetic stirring. Then 10.0 ml H_2O was added to the refluxed mixture, which was extracted with *n*-hexane (3 × 10 ml). The fatty acid methyl ester (2.5 mg and 2.8 mg, respectively) was isolated by chromatography of the *n*-hexane extract over a Silica gel with *n*-hexane-CH_2Cl_2 (9:1) as solvent.

6. Biological activities

6.1. Chemiluminescence assay for determination of immunomodulation activity

A luminol-enhanced chemiluminescence assay was performed, as described in (Helfand et al., 1982). In brief, whole blood (diluted 1:200) and neutrophils (1×10^7) suspended in Hank's balance salt solution with calcium and magnesium ($HBSS^{++}$) were incubated with 50 μl of each test compounds at concentrations of 3.1–50 μg ml^{-1} for 30 min. Then, 50 μl (20 mg ml^{-1}) zymosan (Sigma Chemical Co. St. Louis, MO) followed by 50 μL (7×10^5 M) luminal (G-9382 Sigma) and then $HBSS^{++}$ were added to adjust the final volume to 0.2 ml. $HBSS^{++}$ was used as a control.

6.2. Cytotoxicity assay

Cytotoxic activities of the compounds were evaluated against the PC3 Caucasian prostate adenocarcinoma cell line by the MTT method according to a reported protocol (Zhao et al., 2008). In brief, freshly trypsinized cell suspensions were seeded in 96-well microtiter plates at densities of $1x10^4$ cells per well with test compounds added from DMSO-diluted stock. After 3 days in the culture, the attached cells were incubated with MTT and subsequently solubilized in DMSO. The absorbance at 550 nm was then measured by using a microplate reader. The IC_{50} is the concentration of agent that reduced cell growth by 50% under experimental conditions, with doxorubicin as the positive control (IC_{50} 1.5 μM).

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